Heterogeneity of Molecular Resistance Patterns in Antimony-Resistant Field Isolates of *Leishmania* Species from the Western Mediterranean Area

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Antimonials remain the first-line treatment for the various manifestations of leishmaniasis in most areas where the disease is endemic, and increasing cases of therapeutic failure associated with parasite resistance have been reported. In this study, we assessed the molecular status of 47 clinical isolates of *Leishmania* causing visceral and cutaneous leishmaniasis from Algeria, Tunisia, and southern France. In total, we examined 14 genes that have been shown to exhibit significant variations in DNA amplification, mRNA levels, or protein expression with respect to resistance to antimonials. The gene status of each clinical isolate was assessed via qPCR and qRT-PCR. We then compared the molecular pattern against the phenotype determined via an *in vitro* sensitivity test of the clinical isolates against meglumine antimoniate, which is considered the reference technique. Our results demonstrate significant DNA amplification and/or RNA overexpression in 56% of the clinical isolates with the resistant phenotype. All clinical isolates that exhibited significant overexpression of at least 2 genes displayed a resistant phenotype. Among the 14 genes investigated, 10 genes displayed either significant amplification or overexpression in at least 1 clinical isolate; these genes are involved in several metabolic pathways. Moreover, various gene associations were observed depending on the clinical isolates, supporting the multifactorial nature of *Leishmania* resistance. Molecular resistance features were found in the 3 *Leishmania* species investigated (*Leishmania infantum*, *Leishmania major*, and *Leishmania killicki*). To our knowledge, this is the first report of the involvement of molecular resistance genes in field isolates of *Leishmania major* and *Leishmania killicki* with the resistance phenotype.

L eishmaniasis is a parasitic disease characterized by three major clinical manifestations: cutaneous, mucocutaneous, and visceral leishmaniasis. The latter can be fatal if left untreated, and few drugs are available to treat the disease. In most regions where the disease is endemic, as antimonials are the most affordable medication, they remain the first-line therapy for treatment of the various clinical manifestations. However, these drugs may cause several serious side effects. Moreover, increasing cases of antimonial-related treatment failure have been reported, in part due to parasitic resistance, which was primarily observed in the Bihar region of India (1, 2). The emergence of treatment failure has prompted the use of alternative molecules, such as amphotericin B, which is highly effective against the parasite, although it can potentially lead to severe side effects. The application of liposomal formulations of amphotericin B has reduced therapeutic side effects; however, the high cost of the medication limits the use of such drugs primarily to some regions in southern Europe where the disease is endemic. More recently, miltefosine, which provides the advantage of oral administration, was introduced in regions of India where resistance to antimony has been reported. Nevertheless, point mutation of a parasite drug transporter molecule has been shown to induce miltefosine resistance *in vitro* (3). In addition to the Bihar region, resistance to antimonials has been observed in Sudan and Iran (4, 5). Indeed, assessing parasite susceptibility to antimonials with reliable tests is critical to provide appropriate treatment.

Currently, *Leishmania* resistance to antimonials is assessed *in vitro* by exposing infected macrophages to various concentrations of the drug (6). Although this technique is time-consuming and not completely standardized, it is still considered the reference method. Indeed, efforts have been made to develop alternative techniques to determine resistance phenotype, and most studies have focused on molecular approaches. In fact, resistant *Leishmania* strains that are selected *in vitro* against several drugs, such as methotrexate, arsenicals, and antimonials, display amplification of extrachromosomal DNA forms termed H circles, which harbor the ABC transporter P-glycoprotein A (PGPA) (7, 8, 9). DNA amplification, gene overexpression, and elevated protein levels have also been displayed for other genes implicated in several metabolic pathways, including primarily drug entry, thiol metabolism, peroxide detoxification, and programmed cell death (10–16). Most of these studies were performed with *in vitro*-selected mutants of various *Leishmania* strains, mainly of the nonpatho-

genic species *Leishmania tarentolae* (9, 14, 16, 17). The few studies conducted on field isolates highlighted concordance with molecular patterns identified within *in vitro*-selected mutants; however, several discrepancies have also been reported (10, 18–22).

In the Mediterranean region, little work has been carried out regarding *Leishmania* molecular resistance patterns, and no study has previously been conducted in the Maghreb. In recent years, cases of therapeutic failure have been reported in the Maghreb, an area where visceral and cutaneous leishmaniasis are endemic and where meglumine antimoniate (Glucantime) is the first-line treatment (23, 24).

The aim of our study was to assess the involvement of reported antimonial resistance genes in *Leishmania* clinical isolates from Tunisia, Algeria, and southern France and their correlation with the resistance phenotype determined using the reference technique. This work represents the first assessment of the involvement of resistance genes on a panel of clinical visceral and cutaneous leishmaniasis isolates derived from the Mediterranean region.

MATERIALS AND METHODS

Clinical isolates. Forty-seven clinical isolates were harvested from patients diagnosed with cutaneous leishmaniasis (CL) (18 isolates) or visceral leishmaniasis (VL) (29 isolates) from the Maghreb (26 isolates) and southern France (21 isolates). Clinical isolates derived from the Maghreb were identified at the species level by either isoenzyme analysis performed at the Pasteur Institute of Algeria or matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry performed at the parasitology department of La Timone hospital in Marseilles, France (25, 26). French isolates were identified at the species level by isoenzyme analysis in the French National Reference Center for Leishmaniasis in Montpellier (25). Among these isolates, 38 were *Leishmania infantum* (29 VL and 9 CL), 7 were *Leishmania major*, and 2 were *Leishmania killicki* (Table 1).

Parasite culture. Promastigotes obtained from clinical isolates were maintained at 23°C in Novy-McNeal-Nicolle (NNN) medium and RPMI 1640 medium (500 ml, 1×; reference 52400-025; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 17% heat-inactivated fetal calf serum (HIFCS) (fetal bovine serum; 100 ml; reference 10270-098; Gibco-Invitrogen, Life Technologies) and antibiotics (penicillin, 5,000 U/ml; streptomycin, 5,000 μg/ml; 100 ml; reference 15070-063; kanamycin sulfate, 100×; 100 ml, reference 15160047; Life Technologies). Promastigotes were subcultured every week in NNN medium and every 5 days in RPMI-HIFCS medium. For *in vitro* sensitivity tests, parasites were maintained in RPMI-HIFCS for 5 days and then subcultured in acidified Schneider's medium (Schneider's insect medium; 500 ml; Sigma-Aldrich, St. Louis, MO, USA) for 24 h to facilitate metacyclogenesis as described previously (27).

Parasite lysis and nucleic acid extraction. Three milliliters of 5-day promastigotes (mid-log growth phase) culture in RPMI-HIFCS was centrifuged at 2,000 × g for 10 min. The pellet was resuspended in 1.5 ml of lysis buffer (NucliSENS easyMAG lysis buffer; reference 280134; bioMérieux, Marcy l'Etoile, France) before performing nucleic acid extraction. Sample lysates (800 μl) were subjected to nucleic acid extraction using NucliSENS easyMAG technology (bioMérieux); 140 μl of silica was added to each sample, and the final elution volume was 100 μl. The NucliSENS easyMAG technique allows the simultaneous extraction of both DNA and RNA.

Molecular targets. We analyzed 14 genes involved in *Leishmania* resistance to antimonials (reviewed in reference 28): those encoding aquaglyceroporin (*AQP1*), multidrug resistance (*MDR1*), multidrug-related protein A (*MRPA*) (formerly known as *PGPA*), gammaglutamyl cysteine synthetase (*GSH1*), trypanothione reductase (*TRYR*), tryparedoxin peroxidase (*TRPER*), heat shock protein 83 (*HSP83*), 14-3-3 protein (*14-3-*

3), small kinetoplastid calpain-related protein (*SKCRP*), mitogen-activated protein kinase (*MAPK*), histone 4 (*H4*), arginosuccinate synthetase (*ARGG*), kinetoplastid membrane protein 11 (*KMP11*), and protein 299 (*P299*). For each gene, the target sequence was located in the coding region. Primers and probes were designed using the Primer 3 program, ensuring that they hybridized to gene sequences common to the 3 *Leishmania* species investigated in our study. The melting temperature (T_m) of the primers was 53°C, and the T_m of the probes was 10°C higher than that of the primers to enable probe annealing prior to primer hybridization (Table 2).

RNA purification and reverse transcription. The nucleic acid eluates were treated with DNase (Turbo DNA-free DNase treatment and removal reagents kit; Ambion Inc., Austin, TX, USA) to remove DNA, DNase, and divalent cations from the RNA preparations. Briefly, 1 μl of Turbo DNase and 5 μl of DNase buffer were added to 50 μl of the extraction sample and incubated for 20 min at 37°C. Next, 5 μl of DNase inactivation reagent was added to the sample, which was subsequently mixed and incubated for 5 min at room temperature. The sample mix was then centrifuged at 10,000 × g for 2 min, and the supernatant containing RNA was transferred to a new tube. We then verified that the RNA solution was free of any residual DNA by performing a PCR assay prior to reverse transcription.

One-step reverse transcription was performed using TaqMan reverse transcription reagents (Applied Biosystems, Roche Molecular Systems Inc., Branchburg, NJ, USA). RNA (10 μl) was added to 40 μl of PCR mix containing MgCl₂, deoxynucleoside triphosphates (dNTPs), reverse transcription (RT) buffer, random hexamers, and reverse transcriptase, and reverse transcription was performed using the following cycling conditions: 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. Random hexamers were used instead of poly(A) oligonucleotides to ensure equivalent reverse transcription efficiency of all targets localized on the same polycistronic RNA strand.

Quantitative real-time PCR assay. For each molecular target, specific primers and probe were added to the LightCycler master mix (LightCycler480 probe 2× master mix; Roche Diagnostics GmbH, Mannheim, Germany). Sample DNA or cDNA (1 μl) was added to 24 μl of the PCR mix solution. The following cycling conditions were utilized: *Taq* polymerase activation at 95°C for 10 min and 50 cycles of 10 s of denaturation at 95°C, 30 s of annealing at 53°C, and 10 s at 72°C.

Relative quantification. The DNA polymerase gene (*DNApol*) was selected for relative quantification purposes, as this gene is present in a single copy and displays stable mRNA levels in *Leishmania*. For normalization purposes, we designed 4 plasmids containing a single copy of *ARGG*, *GSH1*, and *HSP83* for the first plasmid, *P299*, *H4*, *SKCRP*, and *MAPK* for the second plasmid, *TRPER*, *MDR1*, and *MRPA* for the third plasmid, and *AQP1*, *TRYR*, *KMP11*, and *14-3-3* for the fourth plasmid. Additionally, each plasmid contained a single copy of *DNApol*. Relative quantification data were calculated with LightCycler 480 software using the $\Delta\Delta C_T$ method as follows: $\Delta\Delta C_T = (C_{T\ \text{target (sample)}} - C_{T\ \text{DNApol (sample)}}) - (C_{T\ \text{target (plasmid)}} - C_{T\ \text{DNApol (plasmid)}})$. The LightCycler software incorporates PCR efficiency into the ratio calculation. This PCR efficiency was determined for each gene using the serial dilution method on the basis of a linear regression slope.

Thresholds. Amplification and overexpression thresholds were fixed by taking into account assay reproducibility. We considered that a clinical isolate displayed significant gene amplification when the relative copy number was 2 times higher than the average copy number of the remaining isolates. Meanwhile, we considered that a clinical isolate displayed significant gene overexpression when the relative mRNA levels were 3 times higher than the average relative mRNA levels of the remaining isolates.

***In vitro* sensitivity tests.** THP1 cells (ATCC TIB-202) were maintained in RPMI-HIFCS medium at 37°C in a 5% CO₂ incubator with high relative humidity. THP1 cells were activated with phorbol myristate acetate (Sigma, L'Isle d'Abeau Chesnes, Saint-Quentin-Fallavier, France) and incubated in Lab-Tek chamber slides (Nalge Nunc International, Na-

TABLE 1 Clinical and epidemiological characteristics and sensitivity test results of *Leishmania* clinical isolates[a]

Isolate	Species	Origin (country)	Clinical form	EC$_{50}$ (μg/ml)
L1	*L. major*	Algeria	CL, HIV coinfection	>60
L2	*L. infantum*	Algeria	CL	>60
L3	*L. infantum*	Algeria	VL, relapse	>60
L4	*L. infantum*	Tunisia	VL, relapse	50
L5	*L. infantum*	Algeria	VL	>60
L6	*L. killicki*	Algeria	CL, chronic form	>60
L7	*L. killicki*	Algeria	CL, chronic form	>60
L8	*L. infantum*	Algeria	CL	>60
L9	*L. infantum*	Algeria	CL	45
L10	*L. infantum*	Algeria	CL	15
L11	*L. major*	Tunisia	CL	45
L12	*L. major*	Tunisia	CL	>60
L13	*L. infantum*	Tunisia	VL, responsive	30
L14	*L. infantum*	Tunisia	VL, relapse	>60
L15	*L. infantum*	France	VL, HIV coinfection, treated with AMB, relapse	30
L16	*L. infantum*	France	VL, HIV coinfection, unresponsive	>60
L17	*L. infantum*	France	CL	>60
L18	*L. major*	Algeria	CL	28
L19	*L. major*	Algeria	CL, 2 cures with Glucantime	42
L20	*L. major*	Algeria	CL	>60
L21	*L. major*	Algeria	CL (diffuse CL, 5 cures with Glucantime)	50
L22	*L. infantum*	France	CL	53
L23	*L. infantum*	Algeria	CL	38
L24	*L. infantum*	France	CL	16
L25	*L. infantum*	France	CL	30
L26	*L. infantum*	France	VL, HIV coinfection, relapse	28
L27	*L. infantum*	France	VL, HIV coinfection, death	36
L28	*L. infantum*	Tunisia	VL, unresponsive	>60
L29	*L. infantum*	France	VL, HIV coinfection, unresponsive	44
L30	*L. infantum*	France	VL, HIV coinfection, unresponsive	26
L31	*L. infantum*	France	VL	32
L32	*L. infantum*	France	VL, HIV coinfection treated with AMB, relapse	>60
L33	*L. infantum*	France	VL, HIV coinfection treated with AMB, relapse	18
L34	*L. infantum*	Tunisia	VL, responsive	30
L35	*L. infantum*	France	VL	17
L36	*L. infantum*	Tunisia	VL	45
L37	*L. infantum*	France	VL	>60
L38	*L. infantum*	France	VL	18
L39	*L. infantum*	France	VL, HIV coinfection, relapse	43
L40	*L. infantum*	France	VL, HIV coinfection treated with AMB, responsive	33
L41	*L. infantum*	Tunisia	VL, responsive	52
L42	*L. infantum*	Tunisia	VL, responsive	7.5
L43	*L. infantum*	France	VL, HIV coinfection treated with AMB, relapse	17
L44	*L. infantum*	France	VL, HIV coinfection, relapse	>60
L45	*L. infantum*	France	VL	30
L46	*L. infantum*	Tunisia	VL, responsive	22
L47	*L. infantum*	Tunisia	VL	50

[a] CL, cutaneous leishmaniasis; VL, visceral leishmaniasis; AMB, amphotericin B; EC$_{50}$, 50% effective dose.

perville, IL, USA). After 48 h activation, the chambers were washed with RPMI to remove residual phorbol myristate acetate. Next, 500 μl of RPMI-HIFCS containing parasites was added to the chambers containing activated THP1 cells with a multiplicity of infection of 10 parasites/THP1 cell. After 24 h infection, the chambers were washed to remove the remaining extracellular parasites. Five hundred microliters of RPMI-HIFCS medium containing Glucantime at concentrations of 0, 7.5, 15, 30, 45, and 60 μg/ml was added to the cultures. The chambers were washed with RPMI on day 3 and day 5, and a new Glucantime-RPMI-HIFCS preparation was then added to the cells. After 7 days of incubation at 37°C, the medium was removed before the slides were washed, dried, and stained with Giemsa solution. In addition to control cultures, a day 0 control was included to verify macrophage infection. The ratio of *Leishmania* para-

sites to THP1 cells was determined for each Glucantime concentration. The 50% effective concentration (EC$_{50}$) corresponds to the concentration of Glucantime that reduced the survival of *Leishmania* parasites by half and was calculated after a semilogarithmic transformation to obtain a linear dose-response relationship. Clinical isolates with an EC$_{50}$ of ≥45 μg/ml were considered resistant, while those with an EC$_{50}$ of <45 μg/ml were considered sensitive, as described previously (F. Gambarelli, R. Piarroux, and H. Dumon, presented at the Medical and Veterinary Symposium on Leishmaniasis, Bordeaux, France, 1993).

RESULTS

Exclusion of nonreproducible PCR results. PCR assay reproducibility was achieved in 94.83% of the experiments (1,248/1,316).

TABLE 2 Probe and primer sequences and functions and accession numbers of the molecular targets analyzed in the study

Target and accession no.	Gene	Function(s)	Primer or probe sequence (5′-3′)[a]
Tryparedoxin peroxidase; AF225212	*TRPER*	Parasite defense against oxidative stress via detoxification of peroxides (29)	F: GTGCTGGAGGAGAAACAG R: TACTGCTTGCTGAAGTATCC P: CTTCTCCACGAACTGAAAAGCCTC
Arginosuccinate synthetase; XM_001465670	*ARGG*	Parasite virulence (30)	F: CACGAAGTCTGACCTGTACTC R: CTCCACACTCGACATTTTTCT P: AGTACGTGACGGTCCAGTTCGAG
Small kinetoplastid calpain-related protein; XM_001687634	*SKCRP*	Associated with calpain-related proteins; key effector of PCD (31, 32)	F: TGTCTGAGATCAAATACGAGAA R: GTCTTTTTGCATTGTAGTGTTG P: AACGGGCTGCTGTTCCGCAT
Mitogen-activated protein kinase; XM_001682543	*MAPK*	Involved in parasite virulence via intracellular proliferation and flagellar morphogenesis; also involved in the apoptotic pathway of the parasite (33, 34)	F: GACCTGAAGCCAGAGAACTT R: AGGTAGAGCTCAGCAAAGAT P: GTACCACCGTGTCGACACGTACTC
Aquaglyceroporin; EU191226	*AQP1*	Membrane channel involved in trivalent antimony uptake (35)	F: TGGCTATTGGACTACTGTTG R: GGTGGAATGATGAAAAAGAC P: TCAGAGCCTTTCACGTTGTACAGC
Gammaglutamylcysteine synthase gene; XM_001464941	*GSH1*	Gammaglutamyl cysteine synthase (γ-GCS), the rate-limiting enzyme of glutathione synthesis, a precursor of trypanothione (36)	F: CAACTAAACATTGACCTGACTC R: AAGTACTGTGCGTAGTAGTAGAGC P: CAGGAACATGACCGACGAACTCTA
Multidrug-related protein A; X17154	*MRPA*	ABC transporter involved in sequestration of the trivalent antimony-thiol complex into an intracellular organelle (37)	F: GAGGGTGTGCAGATGCGGTA R: CATGAACGTCAGCAGCAGCG P: CTTCCCACTCCCTGTCCGCCCGA
Protein 299; XM_001681039	*P299*	Unknown (38)	F: CAACTACGGACAGTTCTTCAA R: TCCATCTAAGCTCACATAATTG P: CGACACAGCCAAGCAGCTCATC
Trypanothione reductase; XM_001462961	*TRYR*	Involved in the reduction of the disulfide form of trypanothione in order to maintain the cellular redox condition; possibly inhibited by trivalent antimony (39)	F: CATCAACGAGAGCTACAAGA R: AATGAGGATGTACTCCGTGT P: GGAGGGCCTCAGCTTTCACA
Heat shock protein 83; XM_001685708	*HSP83*	Prevents programmed cell death activation (40)	F: GCTGATCATCAACACCTTCTA R: GTCAGTGTCTTGTTCTCCTTGT P: GAACAAGGAGATCTTCCTGCGC
Protein 14-3-3; XM_001681451	*14-3-3*	Able to bind to phosphorylated proteins involved in several cellular processes including apoptosis (41)	F: CGCCTACAAGTACATTATCAGT R: GTACTTGTCCAGAAGCTCCA P: CCTCTTCAATGGCTTCCAGCAT
Kinetoplastid membrane protein 11; XM_003722568	*KMP11*	Major immunoreactive membrane glycoprotein (42)	F: GGAAGATGCAGGAACAGAAC R: AACTTCTGCTTGAAGTGCTC P: AGGAGCACTACGAGAAGTTCGAGC
Multidrug resistance; L01572	*MDR1*	Drug efflux (43)	F: GCGACCTGAACCTGACGATC R: GCCCGATCATCGACGACTTG P: CGCACCCAGACGACCCAGAGAACG
Histone 4; XM_001469568	*H4*	Coupled to parasite DNA replication and cellular growth; role in antimony resistance has not been established (44)	F: CGAGGTCTACGAAGAGGTG R: GTAGCCGTAGAGGATGTGG P: ACGTGGAGGACATTGTGCGCT

[a] F, forward primer; R, reverse primer; P, probe.

The assays that failed to display reproducibility either for DNA (1%) or cDNA (9.2%) analyses were excluded from the study. Concerning the DNA analysis, 1 *KMP11*, 3 *MRPA*, and 3 *TRPER* results were excluded from the analysis. Regarding the cDNA analysis, 1 *SKCRP*, 2 *MDR1*, 2 *ARGG*, 2 *MRPA*, 3 *P299*, 4 *AQP1*, 4 *14-3-3*, 5 *TRPER*, 7 *KMP11*, 7 *TRYR*, 11 *MAPK*, and 13 *HSP83* results were excluded from the calculations.

***In vitro* tests.** *In vitro* tests enabled the classification of the 47 isolates as 24 resistant (R) and 23 susceptible (S) isolates. Among the resistant isolates, 18 were from the Maghreb (75%) and 6 were

TABLE 3 Clinical isolates displaying significant gene amplification and the genes involved in each isolate

Isolate and phenotype[a]	Ratio of gene copy no. to avg copy no.[b]				
	TRPER	MAPK	MRPA	AQP1	KMP11
L6 (R)	3.26	4.86	—	—	—
L7 (R)	5.78	5.26	—	—	—
L9 (R)	2.94	—	—	—	—
L4 (R)	—	—	2.66	—	—
L14 (R)	—	—	2.05	2.18	—
L15 (S)	—	—	—	—	2.12
L16 (R)	—	—	—	—	2.20

[a] R, resistant; S, sensitive.
[b] —, not significant.

from southern France (25%). Eight susceptible isolates originated from the Maghreb (34.8%), while 15 originated from southern France (65.2%). Among the resistant isolates, 16 (66.7%) were identified as *L. infantum*, 6 were *L. major* (25%) and 2 were *L. killicki* (8.3). Among the susceptible isolates, 22 were identified as *L. infantum* (95.7%) and 1 was *L. major* (4.3%).

Gene amplification and gene associations. Among the 47 isolates, 7 displayed significant DNA amplification (6 resistant isolates and 1 susceptible isolate). Gene amplification involved *TRPER* (3 R isolates), *MRPA* (2 R isolates), *MAPK* (2 R isolates), *AQP1* (1 R isolate), and *KMP11* (1 R and 1 S isolate) (Table 3).

Two resistant isolates (L6 and L7) displayed amplification of both *TRPER* and *MRPA*. One resistant isolate (L14) displayed amplification of both *AQP1* and *MRPA*. Three resistant isolates (L4, L9, and L16) and 1 susceptible isolate (L15) displayed a single gene amplification.

Gene overexpression and gene associations. Eight isolates, all of which were classified as resistant, displayed significant overexpression of at least 2 genes. Five other isolates, 3 resistant and 2 susceptible isolates, displayed overexpression of a single gene. The 9 genes found to be overexpressed were *14-3-3* (4 R isolates), *PGP* (2 R and 1 S isolate), *AQP1* (3 R isolates), *MAPK* (2 R isolates), *GSH1* (3 R isolates), *ARGG* (1 R isolate), *P299* (4 R and 1 S isolate), *TRPER* (4 R isolates) and *H4* (4 R isolates) (Table 4). Meanwhile, the 34 remaining isolates (21 susceptible and 13 resistant isolates) displayed no significant gene overexpression.

Two resistant isolates (L4 and L5) displayed overexpression of *MRPA*, *TRPER* and *H4*. Isolate L4 also displayed overexpression of *GSH1*. Two resistant isolates (L6 and L7) displayed overexpression of *GSH1*, *TRPER* and *MAPK*, while isolate L7 also displayed overexpression of *P299* and *ARGG*. Three isolates (L1, L2 and L3) displayed overexpression of *14-3-3* and *P299*, and isolate L1 also displayed *H4* overexpression. Isolate L12 showed *AQP1* and *H4* overexpression. Finally, the remaining isolates (L8, L9, L10, L11, and L13) displayed no gene overexpression association. Genes that displayed either significant amplification or overexpression and the related thresholds are summarized in Fig. 1.

Association between gene amplification and gene overexpression. Concomitant gene amplification and overexpression were observed in 3 resistant isolates. *TRPER* and *MAPK* were simultaneously amplified and overexpressed in 2 isolates, and *MRPA* was simultaneously amplified and overexpressed in 1 isolate. The remaining isolates displayed no association between gene amplification and overexpression.

Correlation between molecular patterns of resistance, species, and geographic area. Five *L. infantum* (13.1%) and 2 *L. kil-*

TABLE 4 Clinical isolates displaying significant overexpression and the genes involved

Isolate and phenotype[a]	Ratio of gene overexpression to avg mRNA level[b]								
	14-3-3	P299	MRPA	GSH1	TRPER	MAPK	ARGG	AQP1	H4
L1 (R)	5.13	6.36	—	—	—	—	—	—	5.46
L2 (R)	3.04	10.82	—	—	—	—	—	—	—
L3 (R)	3.11	3.11	—	—	—	—	—	—	—
L4 (R)	—	—	5.84	4.44	9.04	—	—	—	5.47
L5 (R)	—	—	3.82	—	8.17	—	—	—	4.83
L6 (R)	—	—	—	3.07	5.03	16.13	—	—	—
L7 (R)	—	3.81	—	3.24	15.36	8.82	6.21	—	—
L8 (R)	—	—	—	—	—	—	—	4.05	—
L9 (R)	3.01	—	—	—	—	—	—	—	—
L10 (S)	—	—	5.08	—	—	—	—	—	—
L11 (R)	—	—	—	—	—	—	—	3.42	—
L12 (R)	—	—	—	—	—	—	—	3.11	7.23
L13 (S)	—	3.93	—	—	—	—	—	—	—

[a] R, resistant; S, sensitive.
[b] —, not significant.

licki (100%) isolates displayed significant gene amplification; same genes, *TRPER* and *MAPK*, were involved in the 2 *L. killicki* isolates. Seven *L. infantum* isolates (18.4%), 4 *L. major* isolates (57%), and the 2 *L. killicki* isolates (100%) displayed significant gene overexpression.

Among the clinical isolates that displayed significant gene amplification, 5 were isolated in the Maghreb (3 *L. infantum* and 2 *L. killicki* isolates) and 2 were isolated in southern France. All isolates that displayed significant gene overexpression were isolated in the Maghreb (11 out of 18 resistant isolates and 2 out of 8 sensitive isolates), which included 7 *L. infantum* isolates. In contrast, all southern France isolates (15 sensitive and 6 resistant isolates) displayed nonsignificant gene overexpression.

DISCUSSION

We observed various antimonial resistance gene patterns in *Leishmania* field isolates derived from the Mediterranean region. These molecular patterns, which concerned several metabolic pathways, were almost exclusively observed in field isolates exhibiting a resistant phenotype identified via *in vitro* tests. Among these resistant isolates, 3 isolates harbored synergistic gene associations with either concomitant overexpression of *GSH1* and *TRPER* (L4, L6, and L7) or concomitant overexpression of *GSH1* and *MRPA* (L4). In fact, the *GSH1* gene encodes the enzyme responsible for the synthesis of glutathione, a precursor of trypanothione; the latter provides reducing elements that are involved in peroxide detoxification via *TRPER* (29). Trypanothione also couples with trivalent antimony, and the resulting complex is sequestered into an intracellular organelle via *MRPA* (37). For the remaining resistant isolates that displayed either significant amplification or overexpression, only a single pathway or several unlinked pathways were involved. These pathways were associated with apoptosis, parasite virulence, and drug entry. Therefore, it appears that no predominant gene or metabolic pathway was involved in drug resistance in our panel of clinical isolates. Additionally, while isolates displaying overexpression of at least 2 resistance genes always had *in vitro* resistant phenotypes, isolates that overexpressed a single gene exhibited either a resistant or sensitive *in vitro* phenotype. Overall, our results indicate that molecular resistance in *Leishmania* is multifactorial. These findings are generally in accordance with several multigene studies focused on other areas where leishmaniasis is endemic (45, 46).
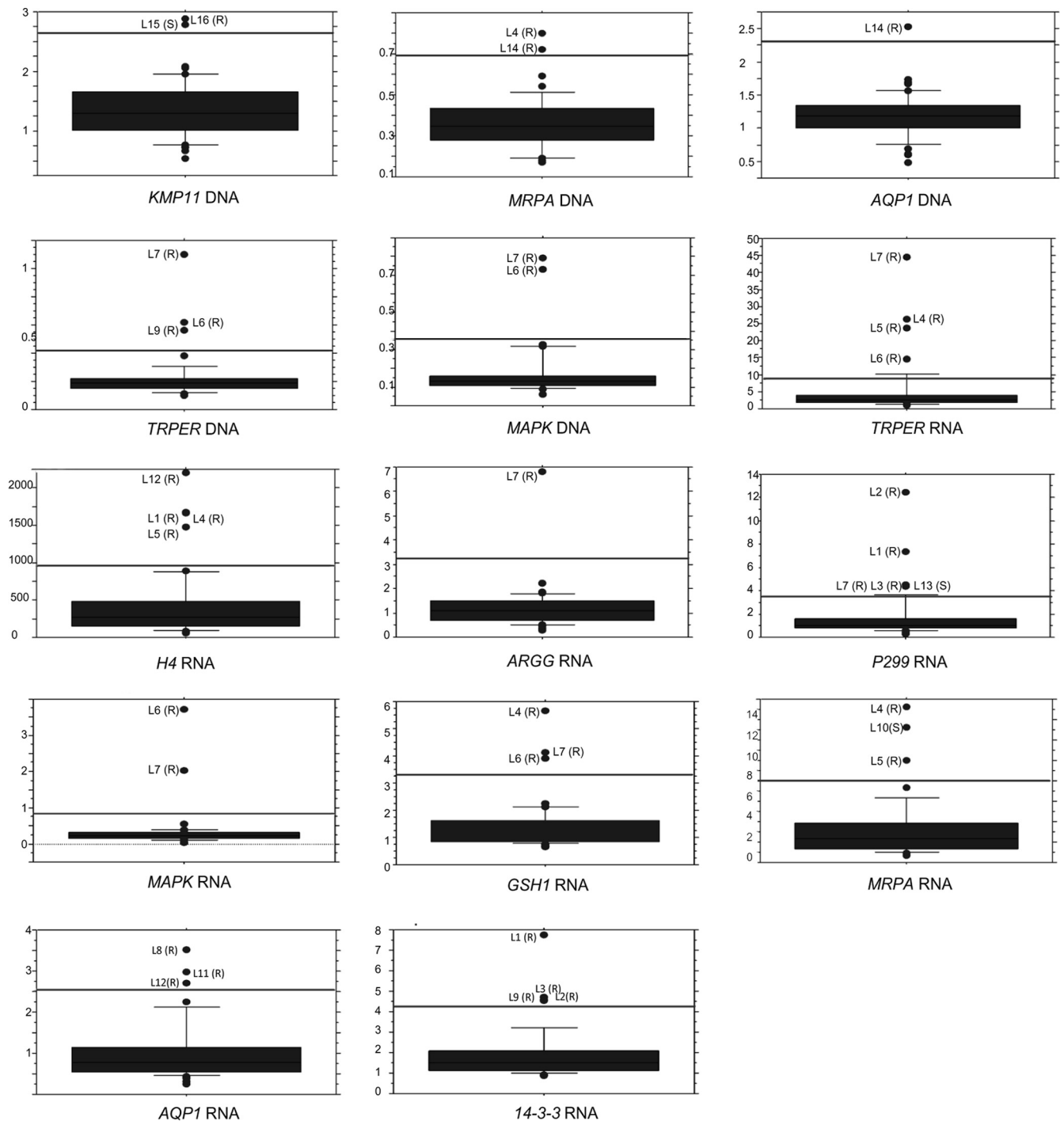
FIG 1 Molecular targets that displayed either significant amplification or overexpression. Lower and upper whiskers correspond to the 10th and 90th percentiles, respectively. The boxes correspond to the interquartile range (between the 25th and the 75th percentiles). The dots outside the whisker interval represent the outliers. Each line above the related box and whisker plot represents the threshold. The identity and phenotype of the clinical isolates that displayed either significant amplification or overexpression are mentioned. R, resistant; S, sensitive.

This study also demonstrates that resistance gene overexpression occurred more frequently than gene amplification, as observed in 11 out of 24 *in vitro* resistant isolates and 2 out of 23 *in vitro* susceptible isolates. Gene overexpression has been reported to occur as a consequence of gene amplification, which was initially described as a major feature associated with resistance at

least within *in vitro*-selected mutants (7). This finding was not confirmed in our series, as gene amplification was rarely concomitant with gene overexpression. Indeed, their association was observed in only 4 isolates. According to Guimond et al., gene overexpression without gene amplification is more likely the consequence of enhanced mRNA stability than transcriptional up-

regulation, as no transcription promoters have yet been identified in *Leishmania* (15). Curiously, we also observed gene amplification in the absence of gene overexpression in 3 isolates. Although the occurrence of gene amplification without gene overexpression has been reported (15, 47), these findings are uncommon, and the significance of this isolated gene amplification remains uncertain.

Various molecular patterns associated with drug resistance identified in our work correlate with those previously described in other surveys. Gene amplification of *MRPA* was observed in 2 resistant isolates in our study. *MRPA* was the first molecular target that displayed gene amplification in *Leishmania in vitro* mutants selected with heavy metals, and this amplification was subsequently demonstrated in resistant field isolates (8–10). The overexpression of *MRPA* observed in 2 resistant isolates in the current study was also described previously (11, 45). *GSH1*, which is generally overexpressed in either *in vitro*-selected mutants or clinical isolates, was overexpressed in 3 resistant isolates (10, 11, 45). Our survey is also in accordance with an earlier study that demonstrated *MAPK* and *H4* overexpression (45). Furthermore, the overexpression of *TRPER*, *ARGG*, *14-3-3*, and *P299* observed in resistant isolates is in accordance with previous proteomic findings (12, 31, 48, 49).

AQP1 gene overexpression was found in 3 resistant isolates, a paradoxical observation considering that *AQP1* plays a role in drug entry. In fact, *AQP1* is a membranous channel through which the trivalent antimony enters the parasite, and *AQP1* overexpression is typically observed in susceptible isolates (11, 45, 47). However, a few cases of *AQP1* overexpression in *L. donovani*-resistant clinical isolates have been reported (18, 19). As the regulation of gene expression in *Leishmania* occurs primarily at the posttranscriptional level, the overexpression of *AQP1* may be modulated at the protein level. Moreover, we cannot exclude the possibility of a mutation of the *AQP1* gene that produces a nonfunctional protein in the resistant isolates displaying *AQP1* overexpression.

We also assessed mRNA downregulation of the tested molecular targets, as certain genes, such as *KMP11*, *SKCRP*, and especially *AQP1*, can be downregulated in resistant isolates (18, 48, 49). However, no significant downregulation of AQP1 or the remaining tested genes was observed (data not shown).

Additionally, neither amplification nor overexpression of *TRYR*, *HSP83*, *SKCRP*, and *MDR1* was observed, although these genes have displayed differential gene numbers or mRNA levels between sensitive and resistant parasites in other studies (19, 45, 46, 49, 50). These negative results could be due to the geographical particularities of mechanisms of resistance in western Mediterranean countries. It may also be due to an insufficient sampling of resistant isolates in our study, as we tested only 24 *in vitro* resistant isolates. Finally, this discrepancy may be the consequence of our choice to fix a relatively high threshold for mRNA levels.

Threshold determination, which was based on assay reproducibility, is indeed crucial for the designation of isolates displaying significant amplification and overexpression. However, defining an appropriate and discriminating threshold is a delicate process. In our study, we verified that the thresholds selected were higher than the mean plus 2 standard deviations (data not shown), thereby allowing a high degree of specificity. In fact, for the 14 genes investigated, 95.6% and 91.3% of the *in vitro* sensitive isolates had values below the gene amplification and overexpression thresholds, respectively. However, our molecular results corre-

lated with the resistance phenotype observed *in vitro* for only 13 out of 24 (54%) resistant isolates. Several studies have defined a significant threshold as a 2-fold increase in RNA levels compared with the levels in a drug-sensitive reference strain (19, 45, 51). Applying such a threshold automatically increases the number of strains exhibiting gene overexpression, although it also introduces the risk of assigning biological significance to measurement variations associated with the technique. Considering the wide variations in repeated measurements of mRNA levels, we preferred to retain a high threshold for our series.

Apart from this high threshold, the absence of significant molecular resistance patterns in almost half of the resistant isolates could be associated with the involvement of other genes not investigated in our survey (10, 11, 15, 20).

Furthermore, although the *in vitro* tests used to classify the strains in the current study are considered the reference method, we acknowledge that some isolates displaying an EC_{50} close to 45 μg/ml were difficult to classify as either sensitive or resistant. Other groups have utilized different thresholds to distinguish between susceptible and resistant isolates, as the reference method has not been completely standardized (20, 22). In the current study, we compared molecular resistance patterns and parasite drug resistance without integrating clinical data. In fact, the interpretation of treatment failure is a complex process, as it may be associated with factors unrelated to the parasite, such as treatment procedure or host immunity. Furthermore, treatment failure criteria have been well established exclusively for visceral leishmaniasis, and no consensus has been reported regarding cutaneous leishmaniasis.

In addition to these technical considerations, it is important to note that all *L. infantum* isolates displaying significant overexpression in our study were isolated in the Maghreb area. However, as only 6 resistant French isolates were assessed in our study, we are unable to make a robust conclusion regarding the variations observed between the two regions. Moreover, the number of isolates tested does not enable us to conclude a significant association between the pattern of molecular resistance and the geographical origin of *L. infantum* isolates, although the findings argue in favor of the presence of resistance factors in the Maghreb area. Interestingly, these resistance factors are not limited to visceral leishmaniasis species, as we identified them in the 3 causative agents of cutaneous leishmaniasis in the Maghreb. To our knowledge, this is the first report of molecular patterns associated with drug resistance in *L. major* and *L. killicki* field isolates.

Overall, as molecular resistance patterns in clinical isolates appear to be heterogeneous and involve numerous genes, it would be challenging to develop suitable molecular methods to replace current time-consuming *in vitro* tests unless new molecular approaches, such as high-throughput whole-genome sequencing, unveil a clear dichotomy between susceptible and resistant isolates. It may be of interest to define subgroups of resistant strains, based on the genes involved in resistance, specific for each geographic area and clinical outcome.

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